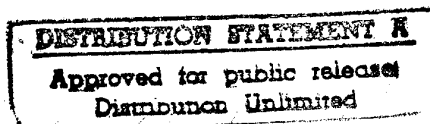# Rapid Assessment of Ceftazidime, Ciprofloxacin, and Gentamicin Susceptibility in Exponentially Growing *E.coli* Cells by means of Flow Cytometry

by

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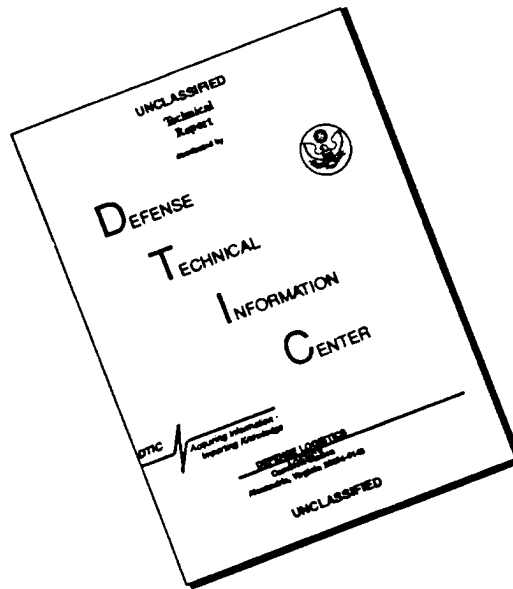
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Abstract Exponentially growing *E.coli* cells were cultivated in the presence of ceftazidime, ciprofloxacin, and gentamicin in concentrations ranging from 1/2 to 8 MIC, permeabilized by means of cold shock in EDTA/Na-azide, stained with the DNA specific dye combination of ethidium bromide and mithramycin before the fluorescence, light scattering, and cell number was measured flow cytometrically. In order to evaluate the applicability of the cold shock procedure, cells were also permeabilized by 70 % ethanol. Permeabilization by cold shock, which eliminates washing of the cells, reduced the preparation time to less than 5 min. A statistically significant increase of the light scattering and fluorescence, i.e. cell size and DNA content, could be detected already after 30 min of ceftazidime and ciprofloxacin exposure, even at sub-MIC concentration. The results obtained with these drugs with cold shock permeabilization were similar to those seen with ethanol fixation. For gentamicin treated cells, however, a majority of the cells lost their fluorescence after cold shock, indicating substantial fragmentation and leakage of DNA by this drug. In gentamicin treated cells fixed in ethanol there was no consistent effect on either light scattering or fluorescence, while cell proliferation was completely inhibited within 30 min of incubation. The present results demonstrate that effects of ceftazidime, ciprofloxacin, and gentamicin on *E.coli* can be detected by flow cytometry within one hour from the beginning of drug exposure to the finished measurement, and with a sensitivity on par with or better than that of conventional plaque assays, which typically require 24 hours or more.

Key words Bacterial drug susceptibility, ceftazidime, ciprofloxacin, gentamicin, flow cytometry, DNA staining, cold shock, ethanol fixation.

INTRODUCTION

Flow cytometry is a technique for measurement of the fluorescence and light scattering of single cells in suspension. The light scattering reflects cellular size and structure while by measuring fluorescence one can determine the cellular content of any constituent which can be labelled with a fluorescent dye. The DNA content is a useful monitor of the growth state of cells and has been used as a marker in several studies of the effects of antibacterial drugs. In some studies DNA associated fluorescence has been measured in combination with light scattering. For cell culture samples light scattering alone has proved sufficient in some studies (6,11,21). However, in clinical samples, the detection of fluorescence may be indispensable in order to distinguish cells from debris and other particulate matter.

For DNA staining of bacteria two obstacles must be overcome, the permeability barrier of the outer membrane and the efflux mechanisms situated in the cytoplasmic membrane (7,8,12). This may be achieved by fixation in 70 % ethanol (1). This procedure, however, includes two steps of centrifugation, leading to increased processing time and loss of cells which reduces the accuracy of the cell counting. We have previously found that exponentially growing *E.coli* cells may be stained efficiently with DNA binding probes after permeabilization by means of cold shock as an alternative to ethanol fixation (7).

Relatively few articles on flow cytometric measurements of antibacterial drug effects have been published. Already in 1982 it was demonstrated that the effect of rifampicin could be detected by measurements of light scattering and DNA content after 10 min of drug incubation (19). In another study similar effects were detected for several other antibacterial drugs as well (18). The drug concentration used in these studies, however, by far exceeded the MIC value of the bacteria. Lower drug concentrations have been employed by others. Thus, Martinez *et al.* showed that effects on *E.coli* DNA content could be detected after 30 min of incubation with beta-lactams, even for sub-MIC concentrations (13). In a recent article published by Durodie *et al.*, it was shown that the protein content/forward scatter ratio plotted as a function of time was a sensitive indicator of the effect of several drugs at the sub-MIC value (5). Within the last few years, several authors have demonstrated effects of drugs at clinically relevant concentrations (2,6,11,14). Probes monitoring membrane potential or binding to nucleic acids have been used in these studies.

In a resent study, we showed that quantitative effects of ampicillin and mecillinam in concentrations of clinical interest could be detected within less than one hour by flow cytometry (21). We wanted to investigate whether similar flow cytometric measurements could reveal effects of other antibiotics and which parameters would be most suited for such measurements.

In order to assess the applicability of flow cytometry for the purpose of drug susceptibility testing in *E.coli*, three different antibiotic drugs were chosen in the present study, each representing a separate class of antibiotics. Thus, ceftazidime, a bacterial wall antagonizer, ciprofloxacin, which affects the DNA gyrase, and gentamicin which among other effects binds irreversibly to ribosomes, were taken to reflect the mode of action of beta-lactams, quinolones, and aminoglycosides, respectively.

It is important to simplify the preparation of cell samples in order to reduce total assay time. Since it has been shown that cold shock permeabilizes exponentially growing bacterial cells (7), the present data include results for cells prepared with ethanol and cold shock in parallel in order to assess the applicability of the latter method for drug treated cells.

MATERIALS AND METHODS
Materials

Ceftazidime was purchased from Glaxo (Middlesex, England) ciprofloxacin from Bayer (Leverkusen, Germany), ethidium bromide (EB), and EDTA from Sigma (MO, USA), gentamicin from Schering-Plough (NJ, USA) and Sigma (MO, USA), mithramycin (Mi) from Pfizer Inc., (N.Y., USA), phosphate-buffered saline (PBS) from Gibco (NY, USA), while Na-azide (azide) was from

Merck (Darmstadt, Germany). Wild type *Escherichia coli* (ATCC 25922) were maintained on luria broth (LB) agar and stored at 4 °C. LB medium (Ph 7.1 and including 0.2 % glucose) was prepared according to Sambrook *et al*. (15). (Tryptone and yeast extract were purchased from Difco (MI, USA)).

MIC determination

The MIC (minimum antibiotic concentration) of each antibiotic was determined by the broth dilution method according to the standard procedure based upon serial two-fold dilutions in LB medium. The MIC value was taken to be to be the lowest concentration of the drug which did inhibit visible bacterial turbidity after over night incubation at 37 °C. The MIC values determined were in agreement with literature data (0.125 μg/ml (ceftazidime), 0.015 μg/ml (ciprofloxacin) and 2 μg/ml (gentamicin)).

Growth and antibiotic exposure

Bacteria were grown in LB medium in well aerated, sterile shaking bottles at 37 °C. Prior to each experiment one *E.coli* colony was transferred to the medium. At exponential growth, i.e. optical density (OD_{600nm}) of 0.2, the culture was diluted 50 fold and allowed to grow further until to OD_{600nm}=0.06. 20 ml aliquots were then transferred into pre-warmed shaking bottles, and the antibiotics were added in the concentrations of 1/2, 1, 2, and 8 MIC. The bacterial density (as measured by flow cytometry) at OD_{600nm}=0.06 was about 5×10^6 bacteria/ml. 100 μl aliquots of the antibiotic-exposed cultures were withdrawn at the appropriate times for OD_{600nm} and flow cytometric analysis. OD_{600nm} was measured with a Shimadzu (UV-160A) spectrophotometer (Shimadzu, Japan).

Bacterial fixation and staining

Cold shock: Following exposure to the antibiotics, the 100 μl samples were immediately mixed into 500 μl of ice cold EDTA (10 Mm) in PBS (Ph 7.4) containing 4 g/l azide. The cells were stained with the DNA specific dye combination of Mi (100 μg/ml) and EB (20 μg/ml) (20). The cells were fully stained after a few minutes on ice. However, for practical reasons, samples were stored on ice up to 3 hr before flow cytometric analysis. The time from sampling and until analysis in the flow cytometer, i.e. a storage period on ice up to 3 hr, did not to affect the results.

Parallel samples were fixed in 70 % ethanol, washed twice and suspended in dye solution according to a procedure described elsewhere (1).

Flow cytometry

Fluorescence, light scattering, and cell number were measured on an Argus 100 flow cytometer (Skatron A/S, Norway) equipped with a 100 W Osram HB 100 mercury arc lamp (Nelex Elektronikk, Oslo, Norway). With each sample $1 - 2 \times 10^4$ cells were analyzed at a typical rate of 500-1000 cells/sec. The excitation wavelength band was 400-440 nm while fluorescence was detected from 470 nm up (B_1-filter block). The low- and high angle light scattering were measured by separate detectors. The measurements were gated by the low angle light scattering. The threshold of the gating was set to eliminate particles (of cellular debris) much smaller than intact cells. For standardization of the measurements monodisperse, fluorescent polymer 1.5 μm diameter beads (Bio-Rad, Hercules, CA, USA) were run at the beginning and end of each experiment. The instrument employs volumetric sample injection from a step motor driven 100 μl precision syringe and thereby facilitates direct measurement of the number of cells per unit volume of sample. Sheath pressure was 0.7 kg/cm². The number of cells measured is represented by the area of the respective histogram peak. Thus, the cell density was calculated from this number, the rate of sample injection, and the duration of the data acquisition.

RESULTS

In the present experiments the fluorescence intensity is a measure of the cellular DNA content

(16). The light scattering and its angular distribution is a complex function of the size, shape, and refractive index of the cell. The scattering to high scattering angles depends more strongly on the refractive index relative to size than does the low angle light scattering (17). Thus, the ratio between low angle and high angle light scattering increases with the ratio between cellular size and dry mass.

Light scattering and fluorescence properties
Ceftazidime and ciprofloxacin: Cells permeabilized by cold shock

Ceftazidime: The low- and high angle light scattering and fluorescence increased with incubation time for cells exposed to ceftazidime at the MIC value (Fig. 1 a-c). There was a significant increase in all the three parameters already after 30 min, most pronounced for the fluorescence (Fig. 1 c). The increase with time for all of the three parameters reflects continued growth in size, dry mass, and DNA content, respectively, of cells unable to complete division, i.e. undergoing filamentation. After 120 min of incubation, the light scattering histograms exhibited two peaks (Fig. 1 a and b). The corresponding low angle light scattering vs fluorescence histogram, Fig. 2, shows four populations. Population I represents cells with growing light scattering and DNA content. II represents disintegrating cells leading to fragments, III, with largely intact content of DNA, which eventually decays leading to debris, IV. For cells incubated with higher drug concentrations, i.e. 2 and 8 MIC, the majority of counts were found in the region IV of Fig. 2, indicating further disintegration (data not shown).

Ciprofloxacin: For cells exposed to ciprofloxacin at the MIC value a similar increase in low- and high angle light scattering and fluorescence with time was observed, Fig. 3 a-c. As with ceftazidime, a significant increase in all of these parameters was evident already after 30 min. Again, the increase at 30 min was most pronounced for the fluorescence (Fig. 3 c). The results are consistent with filamentation and continued synthesis of DNA which was also observed microscopically. Again, two populations developed. Unlike ceftazidime, the corresponding dual parameter histogram, Fig. 4, showed a linear relationship between the low angle light scattering and fluorescence, i.e. between size and DNA content. Cells incubated with higher drug concentration, i.e. 8 MIC, were distributed in four populations, Fig. 5. Population I represents cells which have grown only moderately in size and DNA content. II: filamentous cells, III: leaky cells with reduced content of DNA, and IV: debris. Thus, upon their passage through filamentation to disintegration caused by ciprofloxacin exposure, the cells seem to lose most of their (supposedly fragmented) DNA before finally breaking into debris.

The low angle light scattering and fluorescence of the control cells, i.e. median values from the histograms in Figs. 1 and 3, decreased somewhat with time, Fig. 6 a-b. This decline was observed although the culture was still in exponential growth until at least 60 min after drug addition as judged from the OD_{600nm} data (Fig. 7). This phenomenon appeared to be caused by depletion of the growth medium (Boye, Steen, Skarstad 1983). Fluorescence and light scattering declined further with time, i.e. from 60 min upwards, as the culture approached stationary phase.

The light scattering and fluorescence continued to increase with time for the entire period of observation for the cells that remained intact after treatment with ceftazidime and ciprofloxacin, Fig. 6 a-b. The effects increased with the concentration of the drugs. Even the sub-MIC concentration had a significant effect on both parameters. For both drugs the effect obtained for 1/2 MIC was about half that found for cells exposed to 8 MIC. The magnitude of the increase differed for the two drugs. Thus, cellular size and DNA content of cells exposed to 8 MIC ceftazidime increased more than 10 fold within 120 min, as compared to about half that value for ciprofloxacin. For ciprofloxacin, the largest increase was obtained for 1 MIC, while a somewhat lower increase was obtained with higher concentrations, supposedly due to growth inhibition and accelerated cell degradation.

For both drugs the ratio between low- and high angle light scattering, which may be taken as an indicator of cell size relative to dry weight, increased with time in drug treated cells exposed to cold shock, Fig. 6 c. Again, the effect increased with dose.

Cells permeabilized by ethanol fixation

For both drugs, similar histograms were obtained for cells fixed in ethanol compared to cold shock (histograms not shown). Fig. 8 shows the median value plots for low angle light scattering, fluorescence, and low/high angle light scattering ratio versus time. The results were generally similar to those obtained for cold shock (Fig. 6).

Gentamicin: Cells permeabilized by cold shock and ethanol fixation

The corresponding results for gentamicin were not easily reproducible in spite of our attempts to strictly control the experimental conditions. In some experiments only marginal effects were obtained (Fig. 9). The low angle light scattering remained largely constant, Fig. 9 a, whereas the high angle light scattering appeared to fluctuate, Fig. 9 b. Thus, after 30 min, the high angle light scattering had increased about 20 %, whereupon it decreased to about 80 % of that of the control cells after 60 min. With further incubation, i.e. 120 min, the intensity increased again to a value just below that of the control cells. A somewhat similar variation was observed for the fluorescence, Fig. 9 c. With increasing concentration and time, i.e. up to 8 MIC and 5 hr of incubation, respectively, no further effect of gentamicin was obtained (data not shown). However, the fluorescence histogram obtained at 120 min of incubation (Fig. 9 c), exhibited two minor populations located on each side of the main population. Fig. 10 shows that cells exposed to 8 MIC gentamicin for 120 min, accumulated in four different populations. Population I represents intact cells, II: disintegrated cells with a retained amount of DNA, III: leaky cells with a reduced content of DNA, and IV: debris. The data shown in Figs. 9 and 10 were obtained for ethanol fixed samples. Gentamicin treatment followed by cold shock caused most of the DNA to leak out of the cells. This was verified by fluorescence microscopy. Since gentamicin did not cause a consistent increase in neither light scattering nor fluorescence, the median intensity plots are not shown. In other experiments with gentamicin more pronounced effects of the drug were apparent as broad histograms with several peaks (not shown). However, a significant reduction of cell proliferation was consistently obtained in all experiments, confirming drug activity. Substitution with gentamicin from another manufacturer did not increase the reproducibility of these experiments.

Cell number and optical density

All the drugs led to complete arrest in cell division within 30-60 min of incubation, Fig. 8. At longer times, the cell number decreased, apparently due to cell death and disintegration, Fig. 7 b and c. The onset of this decay was earlier and its magnitude larger with higher drug concentrations. For ceftazidime and ciprofloxacin the effect on OD_{600nm} was hardly detectable until 120 min after drug addition, Fig. 7 a. For gentamicin, however, a significant relative reduction of OD_{600nm} was evident already after 30 min.

DISCUSSION

In the present work we have studied the quantitative effects of ceftazidime, ciprofloxacin, and gentamicin on four essential cell parameters at the single cell level as functions of incubation time and drug concentration by means of flow cytometry. The present results demonstrate that, taken together, the four independent parameters measured, i.e. DNA-associated fluorescence, low- and high angle light scattering, and cell number, provides detection of the drug responses at the MIC values as early as 30 min after drug addition (Figs. 1, 3, 8, and 9).

Antibiotic effects on bacterial single cell parameters have been reported by several authors (Cohen and Sahar 89, Steen et al. 82, Boye et al. 83), although only a few have applied drugs in concentrations of clinical interest (Gant et al. 93, Martinez et al., Mason et al. 94, Ordonez & Wehman, Walberg et al., in press). By measurements of EB associated fluorescence and two light scattering parameters, Gant et al. observed that ampicillin, mecillinam, ciprofloxacin, gentamicin, and cefotaxime affected nucleic acid content and light scattering parameters of E.coli cells. The drug incubation time in these experiments was 3 and 6 hours. The present results show that qualitatively similar effects can be detected as early as within 30 minutes of drug exposure. In agreement with

Gant *et al.*, the present results demonstrate only a slight effect of gentamicin with regard to light scattering and fluorescence compared to the data obtained for ciprofloxacin and cefotaxime. In contrast, gentamicin had a profound effect on cell number and OD_{600nm} already after 30 min. A corresponding effect of ceftazidime and ciprofloxacin on OD_{600nm} could be detected only after 2 hours. Measuring membrane potential, Mason *et al.* detected distinct effects of gentamicin after 30 min (Mason *et al.* 1995). Effects of ampicillin and ciprofloxacin were also detected in that work.

Minimum antibiotic concentration (MAC) is defined as the lowest concentration of an antibacterial drug which affects the bacterial structure, growth rate, or both (Lorian C 1975, Lorian C and Popoola B 1972). Electron microscopy has been used to determine structural changes, while growth inhibition is defined as one log decrease in cell number as measured by plating assay. In agreement with Martinez *et al.* and Durodie *et al.*, the present data indicate that flow cytometry may facilitate rapid detection of the structural changes associated, even with sub-MIC drug exposure, by measurement of morphological changes, i.e. light scattering and fluorescence. The present data indicate that flow cytometry also may be used to detect reduced rate of proliferation associated with sub-MIC exposure. These effects of 1/2 MIC were obviously temporary since turbidity developed in over night cultures.

As seen from the histograms (Figs. 1, 3, and 9), DNA-assocoated fluorescence and light scattering are affected differently by the various drugs, apparently reflecting differences in their mode of action. Such information may be a valuable complement to the mean values plotted in Figs. 6-7, in particular, in the screening of new drugs.

The observed effects of each drug may be explained as follows: The beta-lactam drug, ceftazidime, initially inhibits cell division, while the synthesis of protein and DNA is allowed to continue. Thus, upon drug exposure the light scattering and DNA-associated fluorescence of individual cells begin to increase (Figs. 1 and 6-7) while the cell number remains constant (Fig. 8). This is in agreement with the effect of another beta-lactam antibiotic, ampicillin, described previously (Walberg *et al.*). Since the OD_{600nm} of the cell suspension is the product of cell size and number, it continues to grow at approximately normal rate until disintegration of cells becomes significant or synthesis is otherwise reduced. Thus, the ceftazidime effect on OD_{600nm} becomes evident much later than the changes in the cellular parameters measured by flow cytometry. The ceftazidime treated cells appeared to lose most of their light scattering before the DNA began to decay, indicating that this drug does not cause significant fragmentation of DNA.

Ciprofloxacin acts in a more complex way, leading to the formation of filaments and inhibition of DNA synthesis. Ciprofloxacin inhibits DNA gyrase, leading to accumulation of intermediate-sized DNA fragments (Crumplin GC and Smith JT 1976). The mechanism for formation of filaments is less well understood. In agreement with Gant *et al.*, our results demonstrate filamentation of cells through the increase of the light scattering intensities (Fig. 3 a-b). Apparently, such cells accumulate several fold more DNA (Fig. 3 c), supposedly in fragments. DNA fragmentation is confirmed by substantial leakage of DNA prior to cell disintegration. As evident from Fig. 4, the DNA content of cells exposed to 1 MIC of ciprofloxacin continued to grow at approximately the same rate as the cell size, indicating that on this time scale DNA fragmentation has not commenced. At higher concentrations, however, the results (Fig. 5) clearly indicate substantial leakage of DNA, supposedly as a result of fragmentation.

Gentamicin binds irreversibly to ribosomes. However, the drug has a wide range of additional effects, including alterations in membrane permeability (Day DF 1980). It is well established that permeabilizing of the bacterial wall leads to arrest of DNA replication. It is therefore reasonable to assume that the instantaneous arrest of DNA replication in cells exposed to gentamicin (Fig. 9 c), reflects the permeabilizing action of this drug. The poor staining of gentamicin treated cells exposed to cold shock is probably also associated with its permeabilizing effect which allows DNA to leak out. The effect of gentamicin on the OD_{600nm}, evident already at 30 min, is consistent with inhibition of both cell division and cell growth, i.e. net protein synthesis.

Drug exposure eventually leads to cellular disintegration (Fig. 2, 4, 5, 10, and 11). The route

of disintegration through which the cells pass on their way from intact cells to debris seems to reflect the mode of action of the drug. Ceftazidime treated cells appear to lose their DNA content while the light scattering remains largely unaffected. In contrast, ciprofloxacin treated cells lose most of their light scattering before the DNA begins to decay. Thus, fragmentation of DNA causes a large loss of fluorescence before the main body of the cell begins to disintegrate. In the abscence of DNA fragmentation, on the other hand, a substantial disintegration of the cell body seems to occur prior to the decay of the DNA. The disintegration caused by gentamicin seems to include a combination of these two alternative routes: either the cells lose only their DNA (Fig. 10, peak III) or their light scattering properties (peak II) before finally breaking into debris (peak IV). Since it seems reasonable to assume that only fragmented DNA, in contrast to whole chromosomes, may escape through the permeabilized bacterial wall, these data indicate that gentamicin causes fragmentation of DNA.

In agreement with other observations (6, 11, 21) the present results show that for some drugs, i.e. ciprofloxacin and ceftazidime, the effect of drug exposure can be detected by light scattering alone, i.e. without staining of the cells. However, in clinical samples which may contain large amounts of debris, measurements of light scattering only is likely to be insufficient. Measurement of DNA-associated fluorescence may thus be indispensable to discriminate between cells and other particulate matter. As shown above, the DNA associated fluorescence is in itself an efficient indicator of drug effects.

In general, the present results show that flow cytometry may provide an efficient and rapid assay for detection of the effects of ceftazidime and ciprofloxacin, i.e. cell wall antagonizers and quinolones in E.coli cells. The aminoglycoside gentamicin did not produce similar effects in a consistently reproducible manner. This may be due to the permeabilizing action since the effects of other ribosome targeters have been readily detected by means of flow cytometry (19). It may be concluded that permeabilization by cold shock may facilitate DNA staining as efficiently as ethanol fixation for the detection of beta-lactam and quinolone effects. The use of cold shock substantially shortens and simplifies the sample preparation, thus facilitating a fully automated assay. However, the cold shock preparation does not seem to be applicable in general, at least not for cells exposed to gentamicin.

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FIGURE LEGEND

Fig. 1. The low angle light scattering (a), high angle light scattering (b), and fluorescence (c) histograms of E.coli cells incubated with ceftazidime at the MIC value. The cells were harvested 30, 60, and 120 min after drug addition and permeabilized by cold shock. The low- and high angle light

scattering indicate cellular size and structure, while the fluorescence intensity represents cellular DNA content. The abscissa value (channel number) is directly proportional to the parameter measured on a linear scale. For clarity the histograms have been normalized to a common peak value.

Fig. 2. The low angle light scattering versus fluorescence histogram of cells treated with 1 MIC ceftazidime for 120 min. I: intact filaments, II: disintegrating filaments, III: almost naked chromosomes, IV: debris.

Fig. 3. Low angle light scattering (a), high angle light scattering (b), and fluorescence (c) histograms of *E. coli* cells incubated with 1 MIC ciprofloxacin. The cells were harvested after 30, 60, and 120 min of drug incubation. For details, see Fig. 1.

Fig. 4. The low angle light scattering versus fluorescence histogram of cells treated with 1 MIC of ciprofloxacin for 120 min. For details, see Fig. 1. I: filamentous cells, II: debris.

Fig. 5. The low angle light scattering versus fluorescence histogram of cells incubated for 120 min with 8 MIC ciprofloxacin. For details, see Fig. 1. I: intact cells, II: filaments, III: leaky cells/filaments, IV: debris.

Fig. 6. Median intensity values of the (a) low angle light scattering and (b) fluorescence, and (c) ratio between low- and high angle light scattering (LS), plotted versus time for cells incubated with ceftazidime and ciprofloxacin and prepared with cold shock. The data were obtained from histograms similar to those shown in Figs. 1-2 and from 4 independent experiments. Error bars indicate the full range of values. The ratio between low- and high angle light scattering is an indication between cell size and dry mass.
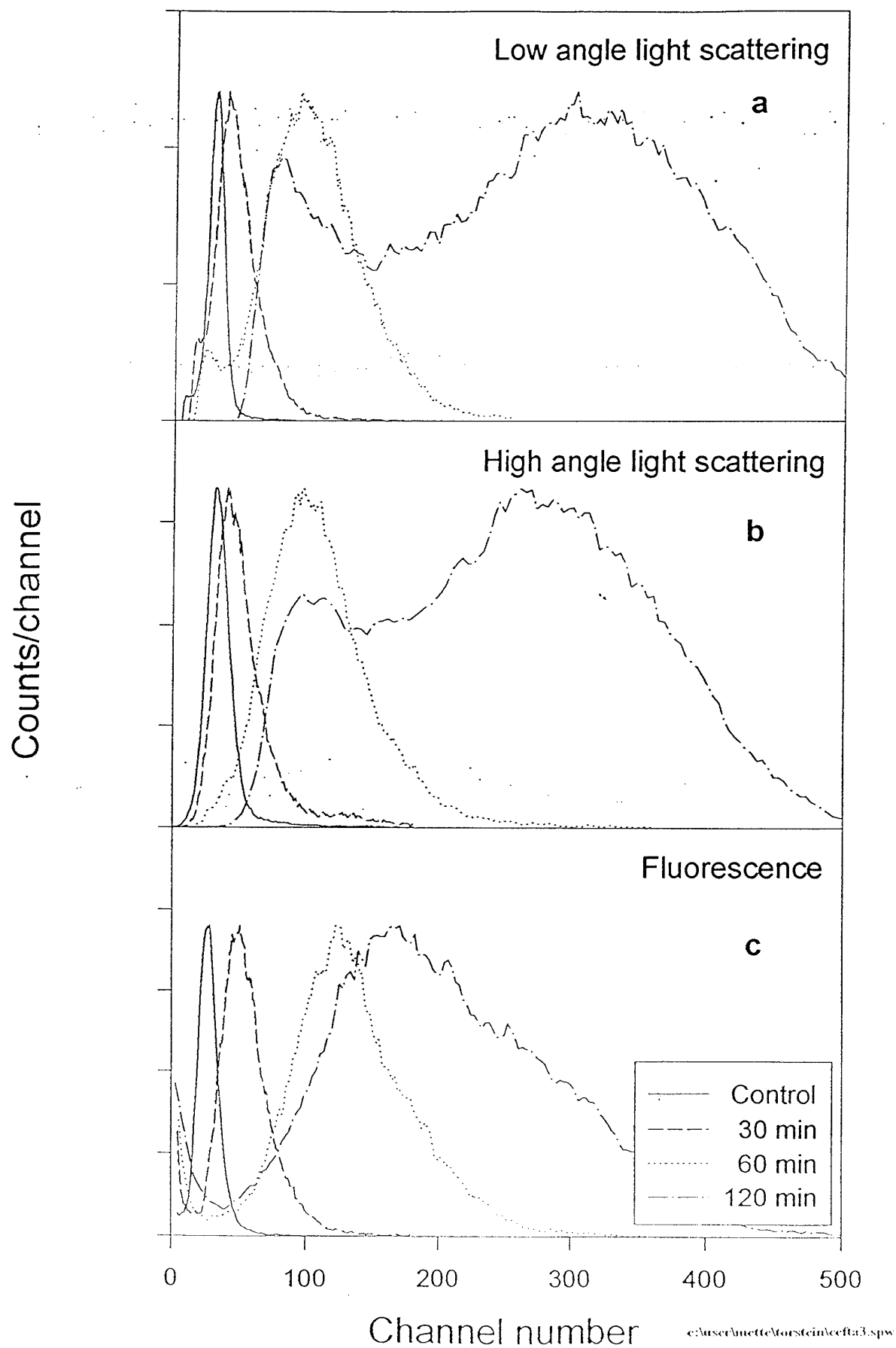
Fig. 7. OD_{600nm} (a) and relative cell number for ethanol-fixed cells (b) and cells prepared by cold shock (c) plotted versus time of incubation with ceftazidime, ciprofloxacin, and gentamicin.
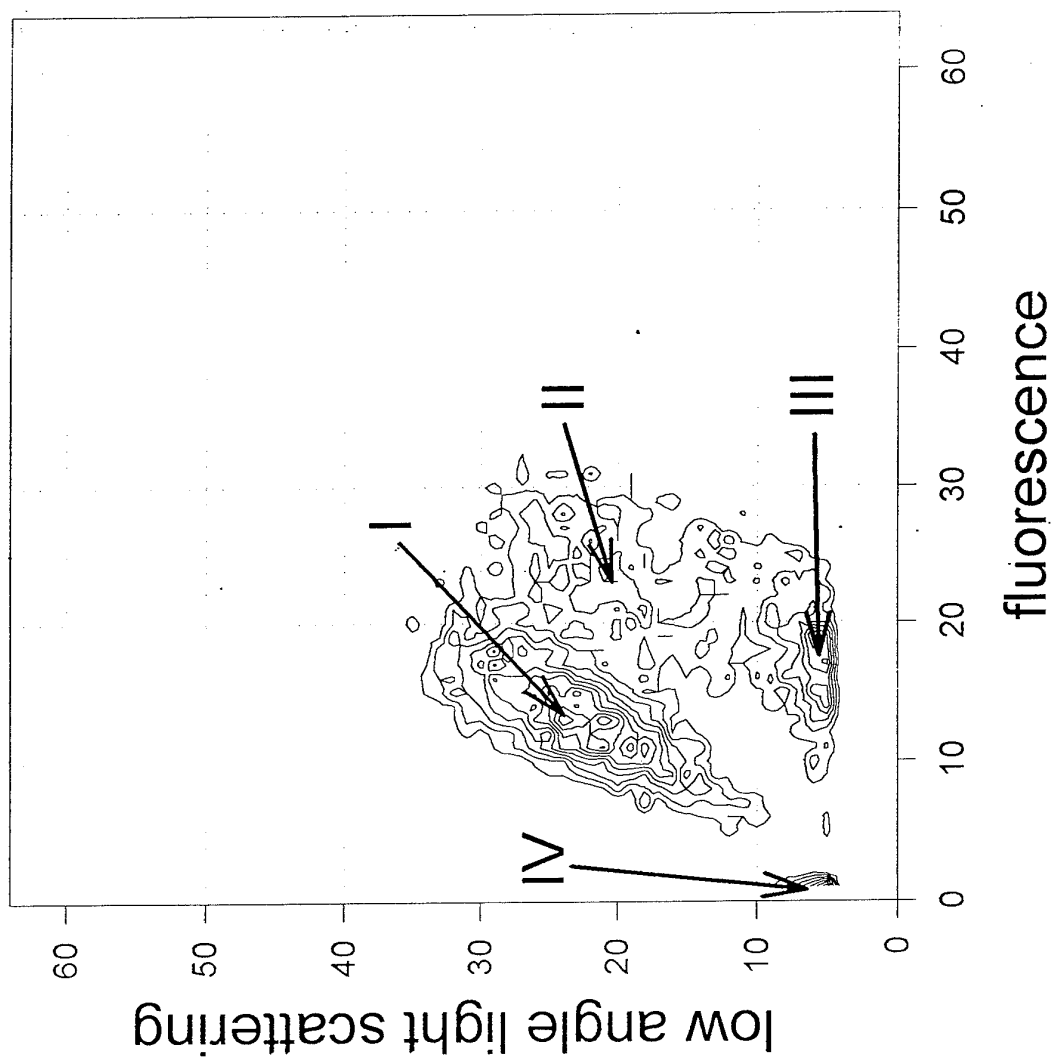
Fig. 8. Data corresponding to those of Fig. 6, however, for ethanol fixed cells.

Fig. 9. The low angle light scattering (a), high angle light scattering (b), and fluorescence (c) histograms of *E. coli* cells incubated with gentamicin at the MIC value. The cells were harvested after 30, 60, and 120 min of drug incubation. The cells were fixed in ethanol prior to staining. Note that the results for gentamicin were difficult to reproduce. Thus, the histograms shown do not reflect the gentamicin effect in general (see text).

Fig. 10. The low angle versus fluorescence histogram of cells incubated for 120 min with 8 MIC gentamicin. The cells were permeabilized by ethanol fixation. I: intact cells, II: leaky cells with a reduced content of DNA, III: disintegrated cells with a retained amount of DNA, III: and IV: debris. III suggests a DNA fragmenting effect of gentamicin, since whole chromosomes are not believed to escape through the permeabilized bacterial wall.
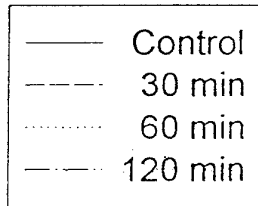
Ceftazidime

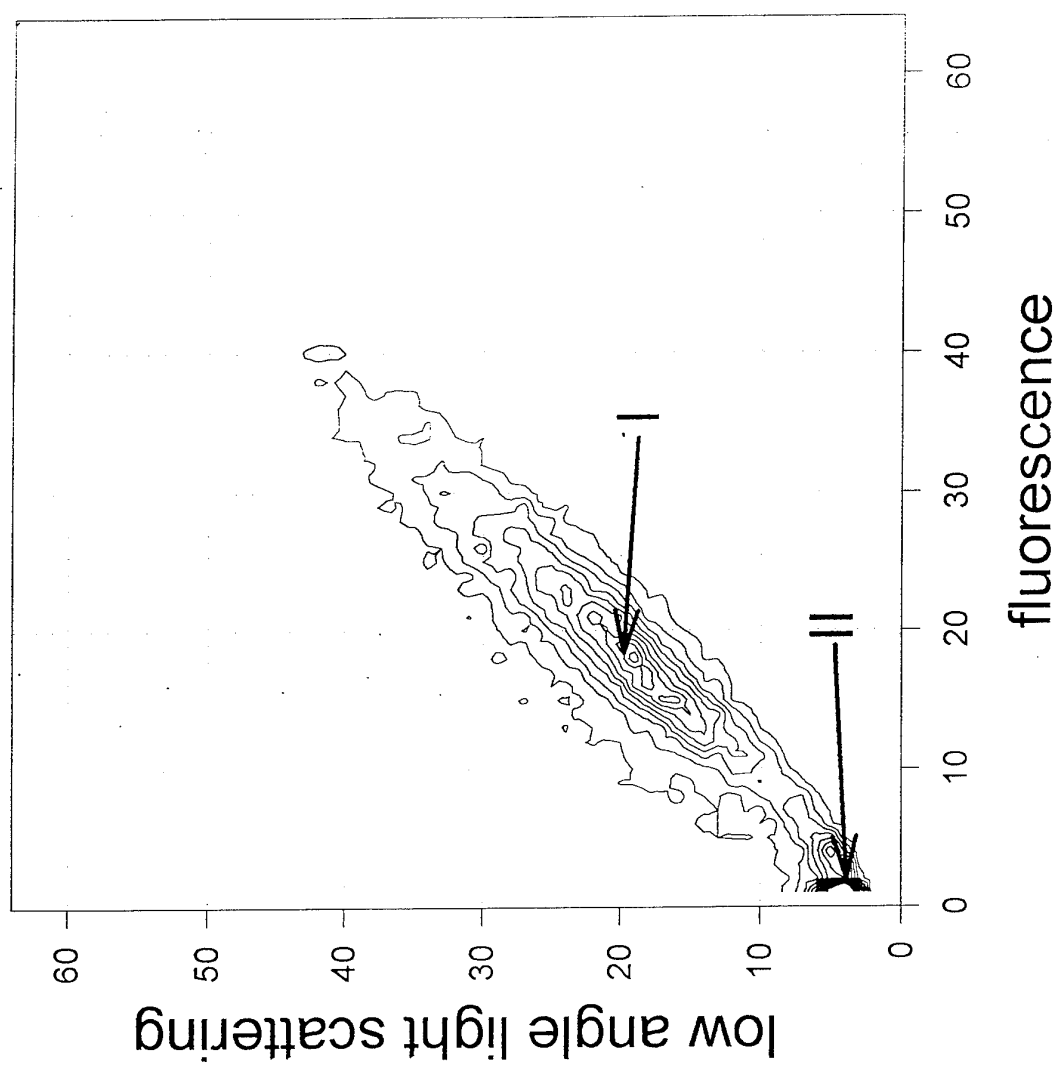Low angle light scattering

a

High angle light scattering

b

Fluorescence
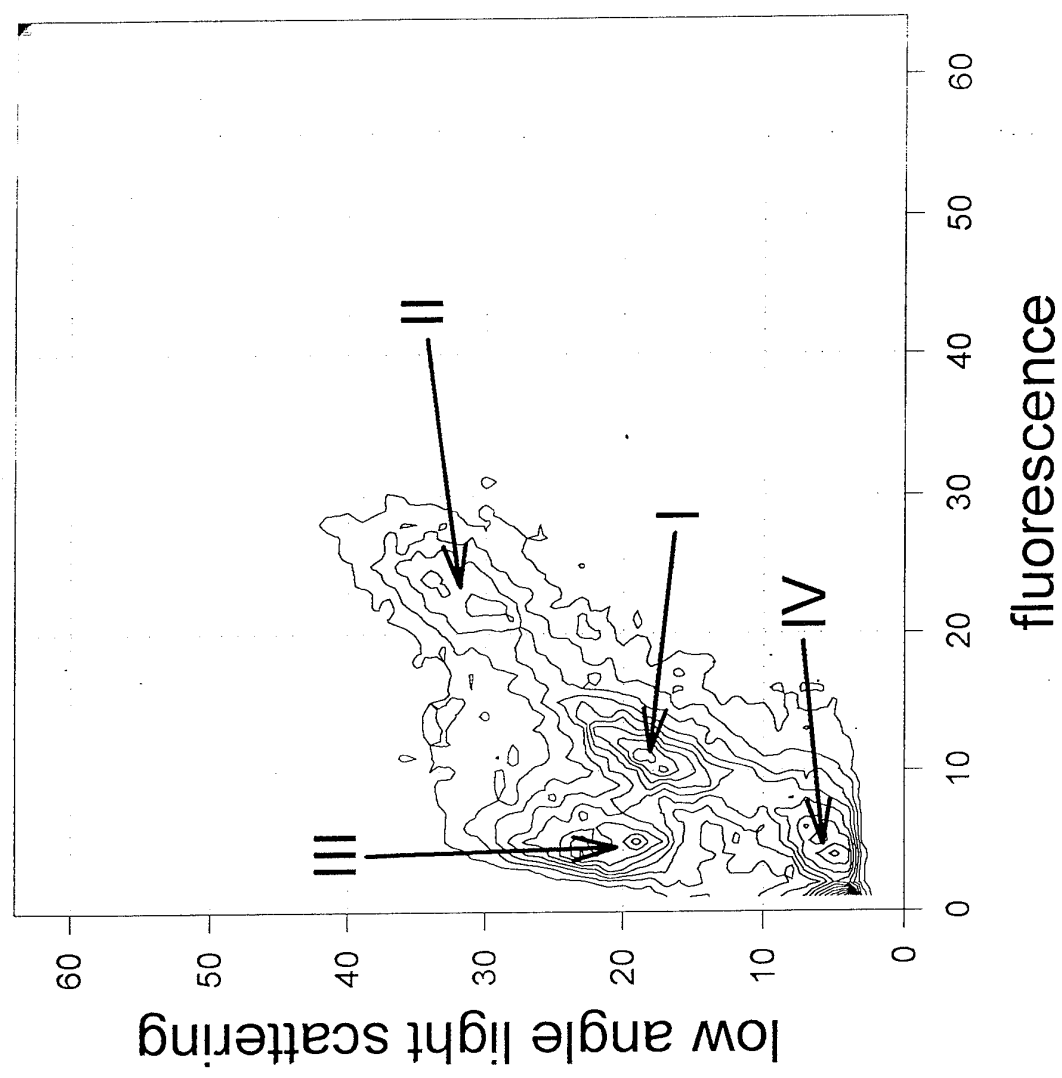
c

	Control
	30 min
	60 min
	120 min

Counts/channel

Channel number

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low angle light scattering

fluorescence

Cipro

Low angle light scattering

a

	Control
	30 min
	60 min
	120 min

High angle light scattering

b

Fluorescence

c

Counts/channel
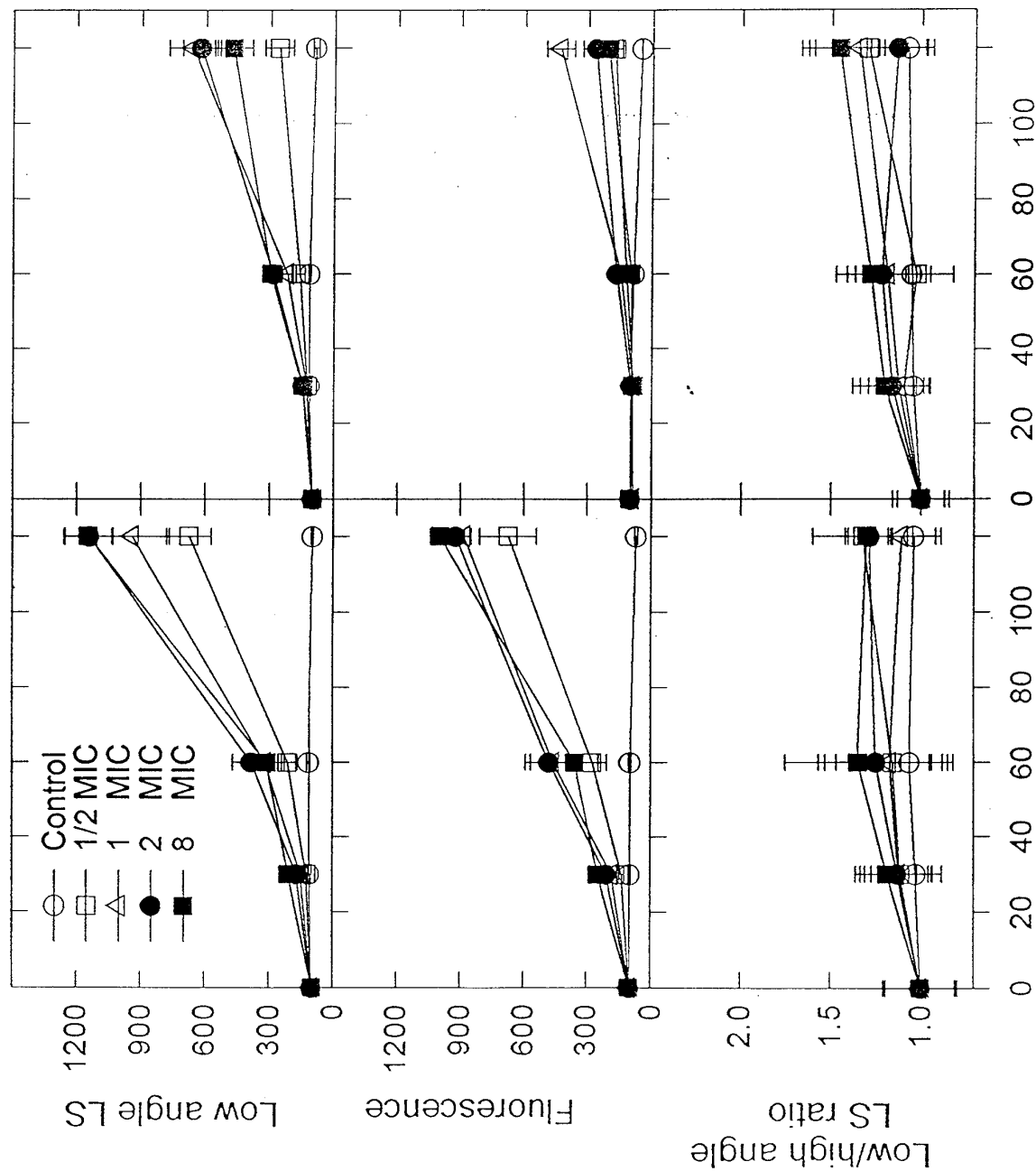
Channel number e:\user\mette\torstein\cipro3.spw

low angle light scattering

fluorescence

fluorescence

low angle light scattering

II

I

IV

III

ceftazidime   ciprofloxacin

a

b

c

Time (min)

\fig\rapallra.spw

Low angle LS

Fluorescence

Low/high angle LS ratio

Control
1/2 MIC
1 MIC
2 MIC
8 MIC

gentamicin   **ciprofloxacin**   **ceftazidime**

a   Ethanol fixation

b

c   Rapid preparation

Time (min)

OD

Rel. cell count

Control
1/2 MIC
1 MIC
2 MIC
8 MIC

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7

ceftazidime

ciprofloxacin

a

b

c

Low angle LS

Fluorescence

Low/high angle
LS ratio

Control
1/2 MIC
1 MIC
2 MIC
8 MIC

Time (min)

\fig\fixallra.spw

Low angle light scattering

a

	Control
	30 min
	60 min
	120 min

High angle light scattering

b

Counts/channel

Fluorescence

c

0   50   100   150   200   250

Channel number

fluorescence

low angle light scattering